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Silencing honey bee (*Apis mellifera*) *naked cuticle* (*nkd*) improves host immune function and reduces *Nosema ceranae* infections

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ABSTRACT

Nosema ceranae is a new and emerging microsporidian parasite of European honey bees, *Apis mellifera* that has been implicated in colony losses worldwide. RNA interference (RNAi), a post-transcriptional gene silencing mechanism, has emerged as a potent and specific strategy for controlling infections of parasites and pathogens in honey bees. While previous studies have focused on the silencing of parasite/pathogen virulence factors, here we explore the possibility of silencing a host factor as a mechanism for reducing parasite load. Specifically, we use an RNAi strategy to reduce the expression of a honey bee gene, *naked cuticle* (*nkd*) which is a negative regulator of host immune function. Our studies found that *nkd* mRNA levels in adult bees were upregulated by *N. ceranae* infection (and thus the parasite may use this mechanism to suppress host immune function), and ingestion of dsRNA specific to *nkd* efficiently silenced its expression. Furthermore, we found that RNAi-mediated knockdown of *nkd* transcripts in *Nosema*-infected bees resulted in upregulation of expression of several immune genes (*Abaecin*, *Apidaecin*, *Defensin-1*, and *PGRP-S2*), reduction of *Nosema* spore loads, and extension of honey bee lifespan. The results of our studies clearly indicate that silencing the host *nkd* gene can activate honey bee immune responses, suppress the reproduction of *N. ceranae* and improve the overall health of honey bees. This study represents a novel host-derived therapeutic for honey bee disease treatment that merits further exploration.

IMPORTANCE

Given the critical role of honey bees in the pollination of agricultural crops, it is urgent to develop strategies to prevent the colony decline induced by the infection of parasites/pathogens. Targeting parasites and pathogens directly by RNAi has been proved to be useful for controlling infections in honey bees but little is known about the disease impacts of RNAi silencing of host factors. Here, we demonstrate that knocking down the gene encoding the honey bee immune repressor *nkd* can suppress the reproduction of *N. ceranae* and improve the overall health of honey bees, which highlights the potential role of host-derived and RNAi-based therapeutics in controlling the infections in honey bees. The information obtained from this study will have positive implications for honey bee disease management practices.

Honey bees, *Apis mellifera*, play a critical role in pollination of important crops. However, the honey bee populations have suffered high losses in much of the world (1), coincident with an increase in agricultural demand for honey bee pollination (2). Specifically, honey bee colony losses in the United States have been exacerbated since the report of Colony Collapse Disorder (CCD), a syndrome that describes large-scale, unexplained losses of managed honey bees (3-9). High levels of parasites and pathogens have been linked to the decline of honey bee colonies (10, 11).

Nosema is a genus of obligate, intracellular microsporidian parasites, which infects many diverse animal species, including honey bees (12, 13). For years, *Nosema* disease of European honey bees was exclusively attributed to a single *Nosema* species - *Nosema apis*. Another species *N. ceranae* was originally detected in Asian honey bees, *A. cerana* (14), and subsequently found to infect European honey bees, *A. mellifera* (15, 16). Since then, the infection of *A. mellifera* by *N. ceranae* has been reported worldwide (17-20), and nosemosis of *A. mellifera* caused by *N. ceranae* is now far more prevalent than that by its native sympatric congener *N. apis* (17, 21-25). Although there are no outward disease symptoms reported (12), *N. ceranae* infection can cause worker bee energetic stress and behavioral changes (26-29), leading to reduced lifespan of infected bees. As an emerging parasite, *N. ceranae* has often been linked to colony losses worldwide. A study based on Spanish honey bee populations showed that natural infection by *N. ceranae* could cause colony collapse (30, 31). New evidence has shown that *N. ceranae* synergistically interacts with pesticides,

92 resulting in more complex and severe diseases in honey bees (32-35). So far, the only
93 registered treatment for nosemosis in North America is fumagillin. With prolonged
94 use of fumagillin, the issues of drug resistance have arisen (36). As a result, novel
95 parasite-specific and environmentally friendly therapeutic options are urgently needed
96 for *Nosema* treatment.

97 RNA interference (RNAi), a post-transcriptional gene silencing mechanism, is an
98 efficient and specific method of gene silencing which functions by inducing
99 degradation of homologous mRNAs (37, 38). RNAi technology has been explored to
100 protect honey bees from infection by pathogens and parasites (39). Three honey bee
101 viruses, *Israeli acute paralysis virus* (IAPV), *Deformed wing virus* (DWV), and
102 *Chinese sacbrood virus* (CSBV) have been successfully inhibited by RNAi under
103 laboratory conditions by feeding bees with virus-specific dsRNAs/siRNAs (40-43).
104 Moreover, a large-scale field application of IAPV dsRNA improved bee survival,
105 colony size and honey yield (44). RNAi has also been used to help control the
106 parasitic mite *Varroa destructor* (45). In one previous study, *N. ceranae* ADP/ATP
107 transporter genes were targeted and silenced by corresponding dsRNAs, resulting in
108 the decline of spore loads and alleviation of diseases in infected bees (46).

109 All honey bee disease applications of RNAi to date have targeted the genes of
110 parasites or pathogens. Nevertheless, disease always involves interactions between
111 hosts and parasites/pathogens, and it is also possible to mitigate infections from the
112 host perspective, i.e. to use RNAi to manipulate host factors that interact with

parasites or pathogens. In fact, *Nosema* infection dramatically alters honey bee transcriptional responses (47-49), providing potential targets for host-based RNAi manipulation.

The Wnt signaling pathway is an important regulator of immune function in mammals (50), and recently has been found to function in regulating immune pathways, specifically Toll pathways, in insects (*Drosophila*) (51). There are several genes that serve as antagonists of the Wnt pathway and thus upregulated expression of these genes should suppress immune function. One of these genes has been found in *Drosophila* - the *naked cuticle* gene (52). As suppressors of immune function, these may serve as excellent targets for parasite manipulation of the hosts' transcriptional pathways - if they parasite can up-regulate these antagonists, it can suppress the hosts immune response. In light of the fact that *nkd* is a negative regulator of Wnt signaling pathway, we hypothesized that *nkd* expression might be regulated by *Nosema* infection and thereby could serve as a potential target of RNAi to mitigate the *Nosema* infection in honey bees. Here we confirmed *nkd* mRNA levels were upregulated when bees were infected by *N. ceranae*. Our RNAi experiments showed that silencing *nkd* led to the enhancement of immune responses through increased immune gene expression, reduction in parasite spore load, and improvement in life expectancy of *N. ceranae* infected bees. We provide unequivocal evidence that silencing the *nkd* gene is an efficient way to control *N. ceranae* infection and improve honey bee health.

METHODS AND MATERIALS

Honey bees. Honey bees used in this study were collected from colonies of *Apis mellifera ligustica* maintained at the USDA-ARS Bee Research Laboratory, Beltsville Maryland. Newly emerged bees were obtained by removing honeycomb frames with sealed brood from strong and healthy colonies that were identified to be *Nosema*-negative, placing the frames into mesh-walled cages individually, and then maintaining the frames in an insect growth chamber at 34±1°C, 55±5% RH overnight. Emerging adult worker bees were collected the next day (< 24 hrs). In order to make sure that the experimental bees were free of *N. ceranae* infection before proceeding to the experimental inoculation, we confirmed the negative status of *Nosema* infection using a hemocytometer and light microscopy. Briefly, 30 abdomens of newly emerged bees were dissected and ground up thoroughly in 30 ml of deionized H_2O. 10 µl of the homogenate was loaded onto a hemocytometer and the presence of spores was determined under light microscopy following a previously described method (53).

Inoculum preparation. *N. ceranae* spores were purified from foragers collected outside the entrance of identified *N. ceranae* infected colonies. Midguts were pulled out and homogenized in sterile, distilled water. The purification of *Nosema* spores from the homogenate was performed as described by Fries *et al*. (2013) (54). The homogenate was filtered through a nylon mesh cloth (65 µm pore size) by centrifuging it for 5 minutes at 3,000 *g*. The supernatant was discarded and the pellet resuspended in sterile water and centrifuged for 10 min at 5,000 *g*, this step was

repeated twice. Finally, the pellet was resuspended in dH_2O and stored at room temperature for no more than one week. The inoculum was obtained by diluting the spore solution with sucrose solution, with a final concentration of 2.0×10^7 spores/ml in 50% (m/V) sucrose solution.

Inoculation. The newly emerged bees were collected and starved for 3 hrs in an incubator (32°C, 75% RH) before inoculation with *Nosema* spores in solution. Individual feeding was performed for each bee by holding two wings of a bee on each side with one hand and feeding the bee with 5 μl inoculum (100,000 spores) with a pipette with the other hand. 30 bees were then distributed into each cup cage, which is a plastic bee-rearing cup with a top-feeder design (55). A 3 ml syringe filled with 50% (m/V) sucrose solution was inserted in the top of the cage to feed the bees and the solution was changed every three days. A small pollen patty was supplied in the bottom of the cage for 6 days. The same number of control bees (without spore inoculation) were transferred into a cup cage as well. There were 4 replicates for each group. All cup cages were maintained in an incubator (32°C, 75% RH). Five bees were sampled from each cage at 6 days, 9 days, 12 days, 15 days, and 18 days post inoculation, respectively. The collected bee samples were immediately put into -80°C and stored until processing.

Production of dsRNA. Primers were designed from the sequence of the *A. mellifera nkd* gene (GenBank accession no. XM_001120899) by using the E-RNAi web service (56), and primers for *GFP* that served as the control gene were used from

176 previous studies (57). All primer sequences were fused with the T7 promoter
177 sequence (underlined; see Table 1). PCR reactions were performed using different
178 templates individually: the cDNA of an adult bee was used for the amplification of
179 *nkd*, and the pGFP vector (Clontech) was used for that of *GFP*. The 100 μl PCR
180 reaction mixture contained the following: 78μl H_2O, 10μl 10× reaction buffer
181 (Invitrogen), 3μl $MgCl_2$, 2μl dNTP mix (10 mM; Invitrogen), 2μl forward primer (20
182 μM), 2μl reverse primer (20 μM), 1μl Taq polymerase (Invitrogen), and 2μl DNA
183 template. The PCR program was 94°C for 3min, followed by 35 cycles of 94°C for
184 30s, 56°C for 30s and 72°C for 90s, and 72°C for 10 min. After each PCR
185 amplification, the products were then verified in 1.0% agarose gels, purified, and then
186 used as the templates for the *in vitro* transcription reaction. The production of dsRNAs
187 was carried out by using the MEGAscript RNAi Kit (Ambion). The transcription
188 reactions were assembled according to the manufacturer's instruction, and the time of
189 incubation at 37°C was extended to 15hrs. The following steps such as nuclease
190 digestion, purification, and elution were performed using the materials associated with
191 the kit. The quality of the dsRNAs was tested using 1.0% agarose gels and their
192 concentration was determined with a Nanodrop 8000 spectrophotometer (Thermo
193 Fisher Scientific Inc.). The products were diluted with sucrose solution to final
194 concentrations of 10μg/ml, 20μg/ml, and 40μg/ml dsRNA in 50% (m/V) sucrose
195 solution, respectively. The final solutions were stored at -80°C until use.

196 **RNA interference treatment.** Before the RNAi treatment, the newly-emerged bees

were collected and inoculated with *N. ceranae* spores as described above, and then transferred into bee rearing cages. In each cage, 20 bees were supplied with 1.5ml of 50% sucrose solution containing *nkd* or *GFP* dsRNA in a 3 ml syringe and a small pollen patty in the bottom of the cage at the same day. Bees were fed with the dsRNA for 15 days; feedings were changed daily. The pollen patty was supplied for the first six days and changed every three days. All cages were incubated at 32°C, 75% RH, and the dead bees were removed every day.

To test the efficiency of gene knockdown, three different concentrations of *nkd* dsRNA solution, 10μg/ml, 20μg/ml, and 40μg/ml, were applied to separate cages, and 20μg/ml *GFP* dsRNA solution was used for control bees. There were three replicates for each treatment. The bees were sampled at 9 days (D9) and 15 days (D15) post the ingestion of dsRNAs and stored at -80 °C until use.

To study the biological responses to the knockdown of *nkd*, 20μg/ml of *nkd* dsRNA and *GFP* dsRNA were fed to the infected bees. Another control group was set up with bees without any treatment and fed only 50% sucrose solution and pollen. Each group contained three replicates. The number of dead bees was recorded daily and were then removed. All the bees were collected at D15 and stored at -80 °C until use.

Spore counting. To evaluate the *Nosema* infection levels of the RNAi-treated bees, the number of spores were counted in individual bees. First, the abdomens were separated, put into 1.5ml Eppendorf tubes individually, and homogenized thoroughly in 1ml dH_2O using a pestle. Then each homogenate was diluted 100 times. 10 μl of

the diluted solution was loaded onto a hemocytometer and the number of spores was counted under light microscopy as described by Cantwell, 1970 (53). The spore load was obtained using the following formula, (spore counts × diluted times) / 20.

RNA extraction and cDNA synthesis. TRIzol reagent (Invitrogen) was used to extract total RNA from the abdomen of individual bees following the manufacturer's protocols. Any genomic DNA contamination was removed by treatment with DNase I (DNA-free kit, Ambion). The purity and quantity of RNA samples were examined by using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific Inc.). All RNAs were stored at -80°C until use. First-strand cDNA was produced from a 20-μl reverse-transcriptional reaction mixture which contained 2 μl total RNA (approximately 1μg/μl), 1 μl dNTP mix (10 mM), 1 μl random primers (0.15 μg/μl), 1 μl DTT (0.1 M), 4 μl 5×First-Strand Buffer, 1 μl SuperScript III RT (200 U/μl, Invitrogen), and 10 μl Nuclease-free water. The reaction program was as follows: 25°C for 5 min, 50°C for 45 min followed by 70°C for 15 min. The cDNAs were stored at -20°C until use.

Quantitative PCR. Quantitative PCR (qPCR) was run on a CFX384 Touch Real-Time PCR System (Bio-Rad, Hercules, CA), and SYBR Green was selected as the detection signal. Primers used here were designed with Primer3 (58) (Table 1). *β-actin* served as the reference gene and all primer pairs were validated as described by (59). Each 10 μl PCR reaction was assembled by mixing 5 μl 2× Brilliant III Ultra-Fast SYBR Green QPCR Mix (Stratagene, La Jolla, CA), 0.25 μl forward

primer (20 mM), 0.25 µl reverse primer (20 mM), 0.5 µl cDNA, and 4 µl nuclease-free water. Each reaction was run in duplicate. The PCR program was 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 60°C for 45 s. Melting curves and no template control (NTC) reactions were monitored to evaluate the quality and specificity of amplification. Only a single peak was seen in all melting curves and no peaks showed in the NTCs. PCR products were run on a 1% agarose gel to confirm the expected amplification sizes. The threshold cycle values (C_t) were generated by the CFX Manager 3.1 (Bio-Rad). The relative quantification of gene expression was calculated with the comparative C_t method (ΔΔCt Method) (60). For each gene, the average Ct value of the target was normalized with the corresponding *β-actin* following the formula: $\Delta Ct = (\text{Average } Ct_{target}) - (\text{Average } Ct_{\beta\text{-actin}})$, and the group of bees with the lowest level of gene expression was chosen as a calibrator ($\Delta Ct_{calibrator}$). The ΔCt value of each group was subtracted from the $\Delta Ct_{calibrator}$ to generate ΔΔCt. The concentration of each target in each group was calculated using the formula $2^{-(\Delta\Delta Ct)}$, and expressed as an n-fold difference relative to the calibrator.

Bioinformatic and statistical analyses. Multiple alignments of insect *nkd* protein sequences was carried out with ClustalX 2.0 (61). Protein domain identification and secondary structure prediction of *A. mellifera nkd* were respectively performed with the InterProScan tool and EMBOSS: garnier algorithm of Geneious v9.1.3 (Biomatters; available from www.geneious.com).

Dynamics of *nkd* gene expression during the course of *Nosema* infection, immune

gene expression, and spore load of infected bees after RNAi treatment were analyzed using independent samples *t*-tests. mRNA levels of *nkd* after dsRNA treatment were analyzed by one-way ANOVA with all compared groups passing an equal variance test, and the post-hoc effects were determined using Tukey HSD tests. Survival analysis was performed using the Kaplan-Meier method, and log-rank and Wilcoxon tests were computed to assess the overall homogeneity between the treatment strata. Pairwise comparisons were carried out using Wilcoxon tests. In all cases, $P < 0.05$ was taken to be significant. All analyses were carried out by PASW Statistics 18 (SPSS Inc.).

RESULTS

Identification of a highly conserved *nkd* gene in honey bees. The identified honey bee *nkd* gene encodes a predicted protein of 545 aa, which shares 92.9% and 89.2% sequence identity with the bumble bee *Bombus impatiens* and stingless bee *Melipona quadrifasciata*, respectively (Fig. 1). This protein is highly conserved in Hymenoptera, as remarkable sequence identity is also seen in non-Apidae species, ranging from 65.5% (*Camponotus floridanus*) to 85.8% (*Megachile rotundata*) (Fig. 1). A conserved EF-hand domain (InterPro ID: IPR002048) - which is known to bind calcium - was identified in the *nkd* proteins (Fig. 1). Additional conserved regions included a region responsible for interaction with *Dishevelled* (*Dsh*), a component of the Wnt signaling pathway (62), and the nuclear localization motif required for nuclear localization and

281 inhibition of Wnt signaling (63) (Fig. 1). Although the diversity of *nkd* protein
282 sequences dramatically increases when expanding the comparison to different insect
283 orders, the sequence features mentioned above are still highly conserved. (Fig. S1)

284 ***N. ceranae* infection up-regulates the expression of *nkd* gene.** As shown in Fig. 2,
285 the dynamics of the *nkd* gene expression was altered during the *N. ceranae* infection.
286 The expression of *nkd* in the infected bees was significantly upregulated compared
287 with that of control bees at Day 6 (D6) and Day 18 (D18) post inoculation (Fig. 2. for
288 D6, *t*-test: $t = -2.774$, df = 9, $P = 0.022$; for D18, *t*-test: $t = -3.387$, df = 10.860, $P =$
289 0.006). Collectively, *N. ceranae* infection upregulated the *nkd* gene expression (*t*-test:
290 $t = -2.872$, df = 63, $P = 0.006$).

291 **Ingestion of dsRNA silences the *nkd* gene expression in *N. ceranae* infected**
292 **bees.** As it is shown in Fig. 3, 9 days feeding of *nkd* dsRNA was insufficient to reduce
293 the mRNA levels of the target gene in adult bees (ANOVA: $F_{1,11} = 0.442$, $P = 0.742$),
294 but the gene expression can be significantly silenced by 15 days ingestion of the
295 corresponding dsRNA (ANOVA: $F_{1,11} = 9.1781$, $P = 0.005$). Moreover, the
296 knockdown of *nkd* exhibited a dosage-dependent manner (Fig. 3): along with the
297 increase of dsRNA concentration, the effect of gene silencing also increased. When 10
298 μg/ml of dsRNA was applied, a 40% knockdown of *nkd* mRNA was achieved, while
299 higher amounts of 20 or 40 μg/ml dsRNA resulted in 50% and 70% gene silencing,
300 and the differences compared to controls became significant (Fig. 3. $P = 0.018$ for 20
301 μg/ml; $P = 0.004$ for 40 μg/ml).

Silencing of the *nkd* gene up-regulates immune gene expression in *N. ceranae* infected bees. As shown in Fig. 4, the *nkd*-silenced bees expressed significantly higher amounts of mRNA for three antimicrobial peptide (AMP) genes *Abaecin*, *Apidaecin*, and *Defensin-1* relative to the control bees (Fig. 4. *t*-test: for *Abaecin*, t = -3.689, df = 9, P = 0.005; for *Apidaecin*, t = -3.047, df = 4.982, P = 0.029; for *Defensin-1*, t = -2.855, df = 9, P = 0.019). Moreover, the expression of a peptidoglycan recognition protein (PGRP) gene, *PGRP-S2* was also significantly up-regulated in *nkd*-silenced bees compared with the control bees (Fig. 4. *t*-test, t = -3.183, df = 5.043, P = 0.024).

Silencing of the *nkd* gene reduces the *Nosema* spore levels and extends the lifespan of *N. ceranae* infected bees. After feeding with *nkd* dsRNA, the spore load of infected bees was significantly (approximately 50%) lower than controls (fed with GFP dsRNA) (Fig. 5. *t*-test: t = 2.458, df = 15.485, P = 0.026), indicating that knockdown of the *nkd* gene expression results in a decrease in *Nosema* infection levels. Survival analysis was performed to further examine the effect of silencing the *nkd* gene on the lifespan of honey bees after *Nosema* infection. The survival distributions for the tested groups were significantly different (Fig 6. log-rank test: χ^2 = 24.472, df = 2, P < 0.001; Wilcoxon test: χ^2 = 25.020, df = 2, P < 0.001). A *Nosema* infection indeed induced higher mortality in the infected honey bees (Fig 6. No treatment vs GFP-dsRNA: Wilcoxon test, χ^2 = 25.218, P < 0.001), however, silencing of *nkd* significantly reduced the incidence of death of adult bees (Fig 6. *nkd*-dsRNA

vs GFP-dsRNA: Wilcoxon test, $\chi^2 = 4.165$, $P = 0.041$).

DISCUSSION

Significant progress has been made in exploring RNAi as a therapeutic strategy for controlling diseases in honey bees, with much attention focused on pathogen-specific virulence determinants. *Nosema* are obligate intracellular parasites and therefore require host cell proteins and pathways to support their replication and many phases of their lifecycles (12, 13). In the present study, the identification and characterization of a host-based factor that is required for parasite pathogenesis in hosts allow us to obtain important insights into the host-parasite interactions and to propose a potential drug target against the parasite.

Wnt signaling is an evolutionarily conserved pathway that plays a critical role in embryogenesis, cell proliferation and differentiation (64). Additionally, recent studies found that activation of the Wnt signaling pathway regulates the immune response to certain pathogenic bacterial infections by up-regulating the expression of anti-inflammatory factors and down-regulating the expression of inflammatory factors (65-68). *nkd* is linked to Wnt signaling and was originally found to act as an inducible antagonist of Wnt signaling during embryonic development in *Drosophila* (52). The results of our study confirmed our hypothesis that *nkd* and Wnt signaling might be also involved in the honey bee responses to infections of *Nosema*. Indeed, *Nosema* infection can result in dramatic host transcriptional responses (47-49), and the

expression of *nkd* gene is significantly upregulated during the *N. ceranae* infection in honey bees, suggesting that *nkd* and Wnt signaling might be targeted by *N. ceranae* during the infection process to alter the defensive function of hosts.

Knockdown of *nkd* by feeding *Nosema*-infected bees with dsRNA specific to *nkd* led to several remarkable alternations, one of which was the modulation of host immune responses. Previous studies have revealed that *N. ceranae* infection induces immunosuppression in honey bees by down-regulating several immune genes such as AMP genes, *Apidaecin*, *Abaecin*, *Defensin-1*, and, *Hymenoptaecin* (69, 70). Our results show that silencing the *nkd* gene in bees infected with *N. ceranae* can reverse immune suppression and enhance the host immune response. The exact mechanism of host immune induction by *nkd* silencing remains unclear. However, silencing of the *nkd* gene ultimately reduces the *Nosema* infection levels and extends the lifespan of infected adult bees.

Since infection always involves the interaction between host and parasite/pathogen, infections can theoretically be controlled by targeting the host of the parasite/pathogen. All previous gene-based efforts to control honey bee parasites or pathogens have targeted these biotic threats directly. Here, by targeting the honey bee *nkd* gene with RNAi, we demonstrate that silencing a honey bee gene can suppress the reproduction of parasites/pathogens and improve the overall health of honey bees. Our results provide a novel host-derived strategy to mitigate honey bee disease. Similar studies have been reported in various species. For example, silencing of the

Cactus gene, an inhibitor of Toll pathway, reduces the extent of dengue virus infection in the midgut by 4 fold in the mosquitos, *Aedes aegypti* (71). Down-regulation of *scavenger receptor class B type 1* (*SR-B1*) expression by RNAi dramatically decreases the susceptibility of human hepatoma cells to Hepatitis C virus (HCC) infection, resulting in the inhibition of this virus infection (72). These studies together indicate that targeting host factors by RNAi can potentially prevent the hosts from infections of parasites/pathogens and promote the overall health of hosts.

RNAi technology has great potential for relieving the impacts of honey bee diseases. Other previous studies (46), combined with our efforts, demonstrate that silencing the parasite/host genes by RNAi manipulation is efficient to suppress parasite development and improve honey bee health to some extent in the laboratory. It is likely that the combination of both strategies, meaning targeting both host and parasite genes in the same RNAi manipulation, will lead to better results for controlling *Nosema* infection. As for field application, more experiments are needed to determine ideal treatment time, the suitable concentration of dsRNA, and other factors. This present study will help direct the application of RNAi to mitigate *N. ceranae* infection in honey bees.

In sum, these studies have identified a host factor required for *Nosema* infection and highlight the potency of host-derived RNAi-based therapeutics to inhibit not only microsporidian parasite infection but also potentially a wide range of pathogens and parasites that cause serious diseases in honey bees.

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REFERENCES

1. **vanEngelsdorp D, Meixner MD.** 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J Invertebr Pathol **103, Supplement:**S80-S95.

2. **Breeze TD, Vaissière BE, Bommarco R, Petanidou T, Seraphides N, Kozák L, Scheper J, Biesmeijer JC, Kleijn D, Gyldenkærne S, Moretti M, Holzschuh A, Steffan-Dewenter I, Stout JC, Pärtel M, Zobel M, Potts SG.** 2014. Agricultural policies exacerbate honeybee pollination service supply-demand mismatches across Europe. PLoS ONE **9:**e82996.

3. **vanEngelsdorp D, Hayes J, Underwood RM, Pettis JS.** 2010. A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. J Apic Res **49:**7-14.

4. **vanEngelsdorp D, Hayes J, Underwood RM, Caron D, Pettis J.** 2011. A survey of managed honey bee colony losses in the USA, fall 2009 to winter 2010. J Apic Res **50:**1-10.

5. **vanEngelsdorp D, Caron D, Hayes J, Underwood R, Henson M, Rennich K, Spleen A, Andree M, Snyder R, Lee K, Roccasecca K, Wilson M, Wilkes J, Lengerich E, Pettis J.** 2012. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. J Apic Res **51:**115-124.

6. **Spleen AM, Lengerich EJ, Rennich K, Caron D, Rose R, Pettis JS, Henson**

M, Wilkes JT, Wilson M, Stitzinger J, Lee K, Andree M, Snyder R, vanEngelsdorp D. 2013. A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. J Apic Res **52:**44-53.

7. **Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS, Rose R, Skinner JA, Tarpy DR, Wilkes JT, vanEngelsdorp D.** 2014. A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. J Apic Res **53:**1-18.
8. **vanEngelsdorp D, Hayes J, Jr., Underwood RM, Pettis J.** 2008. A survey of honey bee colony losses in the U.S., fall 2007 to spring 2008. PLoS ONE **3:**e4071.
9. **vanEnglesdorp D, Underwood R, Caron D, Hayes JJ.** 2007. An estimate of managed colony losses in the winter of 2006–2007: a report commissioned by the Apiary Inspectors of America. Am Bee J **147:**599-603.
10. **Genersch E.** 2010. Honey bee pathology: Current threats to honey bees and beekeeping. Appl Microbiol Biotechnol **87:**87-97.
11. **Ratnieks FLW, Carreck NL.** 2010. Clarity on Honey Bee Collapse? Science **327:**152-153.
12. **Fries I.** 2010. *Nosema ceranae* in European honey bees (*Apis mellifera*). J Invertebr Pathol **103, Supplement:**S73-S79.
13. **Holt HL, Grozinger CM.** 2016. Approaches and challenges to managing

Nosema (Microspora: Nosematidae) parasites in honey bee (Hymenoptera: Apidae) colonies. J Econ Entomol **109:**1487-1503.

14. **Fries I, Feng F, da Silva A, Slemenda SB, Pieniazek NJ.** 1996. *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). Eur J Protistol **32:**356-365.
15. **Higes M, Martín R, Meana A.** 2006. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J Invertebr Pathol **92:**93-95.
16. **Huang W-F, Jiang J-H, Chen Y-W, Wang C-H.** 2007. A *Nosema ceranae* isolate from the honeybee Apis mellifera. Apidologie **38:**30-37.
17. **Chen Y, Evans JD, Smith IB, Pettis JS.** 2008. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J Invertebr Pathol **97:**186-188.
18. **Giersch T, Berg T, Galea F, Hornitzky M.** 2009. *Nosema ceranae* infects honey bees (*Apis mellifera*) and contaminates honey in Australia. Apidologie **40:**117-123.
19. **Higes M, Martín-Hernández R, Garrido-Bailón E, Botías C, Meana A.** 2009. The presence of *Nosema ceranae* (Microsporidia) in North African honey bees (*Apis mellifera intermissa*). J Apic Res **48:**217-219.
20. **Invernizzi C, Abud C, Tomasco IH, Harriet J, Ramallo G, Campá J, Katz H, Gardiol G, Mendoza Y.** 2009. Presence of *Nosema ceranae* in honeybees (*Apis*

460 *mellifera*) in Uruguay. J Invertebr Pathol **101:**150-153.

461 21. **Martín-Hernández R, Meana A, Prieto L, Salvador AM, Garrido-Bailón E,**
462 **Higes M.** 2007. Outcome of colonization of *Apis mellifera* by *Nosema ceranae*.
463 Appl Environ Microbiol **73:**6331-6338.

464 22. **Williams GR, Shafer ABA, Rogers REL, Shutler D, Stewart DT.** 2008. First
465 detection of *Nosema ceranae*, a microsporidian parasite of European honey bees
466 (*Apis mellifera*), in Canada and central USA. J Invertebr Pathol **97:**189-192.

467 23. **Traver BE, Fell RD.** 2011. Prevalence and infection intensity of *Nosema* in
468 honey bee (*Apis mellifera* L.) colonies in Virginia. J Invertebr Pathol **107:**43-49.

469 24. **Yoshiyama M, Kimura K.** 2011. Distribution of *Nosema ceranae* in the
470 European honeybee, *Apis mellifera* in Japan. J Invertebr Pathol **106:**263-267.

471 25. **Martínez J, Leal G, Conget P.** 2012. *Nosema ceranae* an emergent pathogen of
472 *Apis mellifera* in Chile. Parasitol Res **111:**601-607.

473 26. **Goblirsch M, Huang ZY, Spivak M.** 2013. Physiological and behavioral
474 changes in honey bees (*Apis mellifera*) induced by *Nosema ceranae* infection.
475 PLoS ONE **8:**e58165.

476 27. **Higes M, García-Palencia P, Martín-Hernández R, Meana A.** 2007.
477 Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae*
478 (Microsporidia). J Invertebr Pathol **94:**211-217.

479 28. **Mayack C, Naug D.** 2009. Energetic stress in the honeybee *Apis mellifera* from
480 *Nosema ceranae* infection. J Invertebr Pathol **100:**185-188.

29. **Paxton RJ, Klee J, Korpela S, Fries I.** 2007. *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. Apidologie **38:**558-565.

30. **Higes M, Martín-Hernández R, Botías C, Bailón EG, González-Porto AV, Barrios L, del Nozal MJ, Bernal JL, Jiménez JJ, Palencia PG, Meana A.** 2008. How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environ Microbiol **10:**2659-2669.

31. **Higes M, Martín-Hernández R, Garrido-Bailón E, González-Porto AV, García-Palencia P, Meana A, Del Nozal MJ, Mayo R, Bernal JL.** 2009. Honeybee colony collapse due to *Nosema ceranae* in professional apiaries. Environ Microbiol Rep **1:**110-113.

32. **Alaux C, Brunet J-L, Dussaubat C, Mondet F, Tchamitchan S, Cousin M, Brillard J, Baldy A, Belzunces LP, Le Conte Y.** 2010. Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). Environ Microbiol **12:**774-782.

33. **Pettis J, vanEngelsdorp D, Johnson J, Dively G.** 2012. Pesticide exposure in honey bees results in increased levels of the gut pathogen *Nosema*. Naturwissenschaften **99:**153-158.

34. **Vidau C, Diogon M, Aufauvre J, Fontbonne R, Viguès B, Brunet J-L, Texier C, Biron DG, Blot N, El Alaoui H, Belzunces LP, Delbac F.** 2011. Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of

502 honeybees previously infected by *Nosema ceranae*. PLoS ONE **6:**e21550.
503 35. **Wu JY, Smart MD, Anelli CM, Sheppard WS.** 2012. Honey bees (*Apis*
504 *mellifera*) reared in brood combs containing high levels of pesticide residues
505 exhibit increased susceptibility to *Nosema* (Microsporidia) infection. J Invertebr
506 Pathol **109:**326-329.
507 36. **Huang W-F, Solter LF, Yau PM, Imai BS.** 2013. *Nosema ceranae* escapes
508 fumagillin control in honey bees. PLoS Path **9:**e1003185.
509 37. **Hannon GJ.** 2002. RNA interference. Nature **418:**244-251.
510 38. **Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC.** 1998.
511 Potent and specific genetic interference by double-stranded RNA in
512 *Caenorhabditis elegans*. Nature **391:**806-811.
513 39. **Grozinger CM, Robinson GE.** 2015. The power and promise of applying
514 genomics to honey bee health. Curr Opin Insect Sci **10:**124-132.
515 40. **Liu X, Zhang Y, Yan X, Han R.** 2010. Prevention of Chinese sacbrood virus
516 infection in *Apis cerana* using RNA Interference. Curr Microbiol **61:**422-428.
517 41. **Chen YP, Pettis JS, Corona M, Chen WP, Li CJ, Spivak M, Visscher PK,**
518 **DeGrandi-Hoffman G, Boncristiani H, Zhao Y, vanEngelsdorp D, Delaplane**
519 **K, Solter L, Drummond F, Kramer M, Lipkin WI, Palacios G, Hamilton MC,**
520 **Smith B, Huang SK, Zheng HQ, Li JL, Zhang X, Zhou AF, Wu LY, Zhou JZ,**
521 **Lee M-L, Teixeira EW, Li ZG, Evans JD.** 2014. Israeli acute paralysis virus:
522 epidemiology, pathogenesis and implications for honey bee health. PLoS Path

10:e1004261.

42. **Desai SD, Eu YJ, Whyard S, Currie RW.** 2012. Reduction in deformed wing virus infection in larval and adult honey bees (*Apis mellifera* L.) by double-stranded RNA ingestion. Insect Mol Biol **21:**446-455.

43. **Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, Sela I.** 2009. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Mol Biol **18:**55-60.

44. **Hunter W, Ellis J, vanEngelsdorp D, Hayes J, Westervelt D, Glick E, Williams M, Sela I, Maori E, Pettis J, Cox-Foster D, Paldi N.** 2010. Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (*Apis mellifera*, Hymenoptera: Apidae). PLoS Path **6:**e1001160.

45. **Garbian Y, Maori E, Kalev H, Shafir S, Sela I.** 2012. Bidirectional transfer of RNAi between honey bee and *Varroa destructor*: *Varroa* gene silencing reduces *Varroa* population. PLoS Path **8:**e1003035.

46. **Paldi N, Glick E, Oliva M, Zilberberg Y, Aubin L, Pettis J, Chen Y, Evans JD.** 2010. Effective gene silencing in a microsporidian parasite associated with honeybee (*Apis mellifera*) colony declines. Appl Environ Microbiol **76:**5960-5964.

47. **Aufauvre J, Misme-Aucouturier B, Viguès B, Texier C, Delbac F, Blot N.** 2014. Transcriptome analyses of the honeybee response to *Nosema ceranae* and

insecticides. PLoS ONE **9:**e91686.

48. **Dussaubat C, Brunet J-L, Higes M, Colbourne JK, Lopez J, Choi J-H, Martín-Hernández R, Botías C, Cousin M, McDonnell C, Bonnet M, Belzunces LP, Moritz RFA, Le Conte Y, Alaux C.** 2012. Gut pathology and responses to the microsporidium *Nosema ceranae* in the honey bee *Apis mellifera*. PLoS ONE **7:**e37017.

49. **Holt H, Aronstein K, Grozinger C.** 2013. Chronic parasitization by *Nosema* microsporidia causes global expression changes in core nutritional, metabolic and behavioral pathways in honey bee workers (*Apis mellifera*). BMC Genomics **14:**799.

50. **Staal FJT, Luis TC, Tiemessen MM.** 2008. WNT signalling in the immune system: WNT is spreading its wings. Nat Rev Immunol **8:**581-593.

51. **Gordon MD, Dionne MS, Schneider DS, Nusse R.** 2005. WntD is a feedback inhibitor of Dorsal/NF-[kappa]B in *Drosophila* development and immunity. Nature **437:**746-749.

52. **Zeng W, Wharton KA, Mack JA, Wang K, Gadbaw M, Suyama K, Klein PS, Scott MP.** 2000. *naked cuticle* encodes an inducible antagonist of Wnt signalling. Nature **403:**789-795.

53. **Cantwell GE.** 1970. Standard methods for couting *Nosema* spores. Am Bee J **110:**222-223.

54. **Fries I, Chauzat MP, Chen YP, Doublet V, Genersch E, Gisder S, Higes M,**

McMahon DP, Martin-Hernandez R, Natsopoulou M, Paxton RJ, Tanner G, Webster TC, Williams GR. 2013. Standard methods for *Nosema* research. J Apic Res **52**.

55. **Evans JD, Chen YP, Prisco Gd, Pettis J, Williams V.** 2009. Bee cups: single-use cages for honey bee experiments. J Apic Res **48:**300-302.

56. **Horn T, Boutros M.** 2010. E-RNAi: a web application for the multi-species design of RNAi reagents—2010 update. Nucleic Acids Res **38:**W332-W339.

57. **Amdam GV, Norberg K, Page JRE, Erber J, Scheiner R.** 2006. Downregulation of *vitellogenin* gene activity increases the gustatory responsiveness of honey bee workers (*Apis mellifera*). Behav Brain Res **169:**201-205.

58. **Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG.** 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res **40:**e115.

59. **Li W, Huang ZY, Liu F, Li Z, Yan L, Zhang S, Chen S, Zhong B, Su S.** 2013. Molecular Cloning and characterization of juvenile hormone acid methyltransferase in the honey bee, *Apis mellifera*, and its differential expression during caste differentiation. PLoS ONE **8:**e68544.

60. **Schmittgen TD, Livak KJ.** 2008. Analyzing real-time PCR data by the comparative CT method. Nature Protocols **3:**1101-1108.

61. **Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA,**

McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics **23:**2947-2948.

62. **Rousset R, Mack JA, Wharton KA, Axelrod JD, Cadigan KM, Fish MP, Nusse R, Scott MP.** 2001. *naked cuticle* targets *dishevelled* to antagonize Wnt signal transduction. Genes Dev **15:**658-671.

63. **Waldrop S, Chan C-C, Cagatay T, Zhang S, Rousset R, Mack J, Zeng W, Fish M, Zhang M, Amanai M, Wharton KA.** 2006. An unconventional nuclear localization motif is crucial for function of the *Drosophila* Wnt/Wingless antagonist *naked cuticle*. Genetics **174:**331-348.

64. **Logan CY, Nusse R.** 2004. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol **20:**781-810.

65. **Chen K, Yin L, Nie X, Deng Q, Wu Y, Zhu M, Li D, Li M, Wu M, Huang X.** 2013. β-Catenin promotes host resistance against *Pseudomonas aeruginosa* keratitis. J Infect **67:**584-594.

66. **Liu X, Wu S, Xia Y, Li XE, Xia Y, Zhou ZD, Sun J.** 2011. Wingless homolog Wnt11 suppresses bacterial invasion and inflammation in intestinal epithelial cells. Am J Physiol-gastr L **301:**G992-G1003.

67. **Silva-García O, Valdez-Alarcón JJ, Baizabal-Aguirre VM.** 2014. The Wnt/β-catenin signaling pathway controls the inflammatory response in infections caused by pathogenic bacteria. Mediat Inflamm **2014:**7.

68. **Liu X, Lu R, Wu S, Zhang Y-g, Xia Y, Sartor RB, Sun J.** 2012. Wnt2 inhibits enteric bacterial-induced inflammation in intestinal epithelial cells. Inflamm Bowel Dis **18:**418-429.

69. **Antúnez K, Martín-Hernández R, Prieto L, Meana A, Zunino P, Higes M.** 2009. Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). Environ Microbiol **11:**2284-2290.

70. **Chaimanee V, Chantawannakul P, Chen Y, Evans JD, Pettis JS.** 2012. Differential expression of immune genes of adult honey bee (*Apis mellifera*) after inoculated by *Nosema ceranae*. J Insect Physiol **58:**1090-1095.

71. **Xi Z, Ramirez JL, Dimopoulos G.** 2008. The *Aedes aegypti* Toll pathway controls dengue virus infection. PLoS Pathog **4:**e1000098.

72. **Zeisel MB, Koutsoudakis G, Schnober EK, Haberstroh A, Blum HE, Cosset F-L, Wakita T, Jaeck D, Doffoel M, Royer C, Soulier E, Schvoerer E, Schuster C, Stoll-Keller F, Bartenschlager R, Pietschmann T, Barth H, Baumert TF.** 2007. Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. Hepatology **46:**1722-1731.

73. **Mutti NS, Dolezal AG, Wolschin F, Mutti JS, Gill KS, Amdam GV.** 2011. IRS and TOR nutrient-signaling pathways act via juvenile hormone to influence honey bee caste fate. J Exp Biol **214:**3977-3984.

74. **Yang X, Cox-Foster DL.** 2005. Impact of an ectoparasite on the immunity and

628 pathology of an invertebrate: Evidence for host immunosuppression and viral
629 amplification. Proc Natl Acad Sci USA **102:**7470-7475.
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631 **TABLES**

632 **TABLE 1** Primer sequences used in this study.

	Gene (accession no.)	Primer	Sequence (5'→3')	Amplicon location	Amplicon length (bp)	Reference
RNAi	*nkd* (XM_001120899)	nkd-RNAi-F	taatacgactcactatagggcgaCGCGCTTATGTTCAACCTC	1889-2122	234	This study
		nkd-RNAi-R	taatacgactcactatagggcgaGGTCGCGTGTTTCAAATGAT			
	GFP (AF324407)	GFP- RNAi -F	taatacgactcactatagggcgaTTCCATGGCCAACACTTGTCA	173-674	502	(57)
		GFP- RNAi-R	taatacgactcactatagggcgaTCAAGAAGGACCATGTGGTC			
qPCR	*nkd*	nkd-F	AGGATGACGGTGAAAATGCG	1365-1540	176	This study
		nkd-R	ATTAGTCGTGAGGAGAGGCG			
	β-actin (NM_001185145)	actin-F	TGCCAACACTGTCCTTTCTG	1018-1173	156	(73)
		actin-R	AGAATTGACCCACCAATCCA			
	Abaecin (NM_001011617)	Abaecin-F	AGATCTGCACACTCGAGGTCTG	14-214	201	(74)
		Abaecin-R	TCGGATTGAATGGTCCCTGA			
	Apidaecin (NM_001011613)	Apidaecin-F	TTTTGCCTTAGCAATTCTTGTTG	58-137	80	This study
		Apidaecin-R	GCAGGTCGAGTAGGCGGATCT			
	Defensin-1 (NM_001011616)	Defensin-1-F	TGTCGGCCTTCTCTTCATGG	88-288	201	This study
		Defensin-1-R	TGACCTCCAGCTTTACCCAAA			
	PGRP-S2 (NM_001163716)	PGRP-S2-F	TTGCACAAAATCCTCCGCC	146-274	129	This study
		PGRP-S2-R	CACCCCAACCCTTCTCATCT			

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FIGURE LEGENDS

FIG 1 Sequence conservation and predicted secondary structure of *A. mellifera nkd*. (A) multiple alignment of *A. mellifera* (XP_001120899), *Bombus impatiens* (XP_012249347), *Megachile rotundata* (XP_003702467), *Melipona quadrifasciata* (KOX70301), *Atta colombica* (KYM87061), *Harpegnathos saltator* (XP_011151892), and *Camponotus floridanus* (EFN66676) *nkd* protein sequences. Black and grey shadings indicate identity and high conservation of amino acids, respectively. The region responsible for interaction with *dsh* is indicated with the red bar. The nuclear localization motif is underlined by the green bar. The blue box highlights the EF-hand domain. (B) protein domain identification and secondary structure prediction of *A. mellifera nkd*. The conserved domain is shown with a black bar.

FIG 2 The expression profile of *nkd* during *N. ceranae* infection. The X axis indicates the days post inoculation of *Nosema* spores. The relative gene expression levels (Y axis) are expressed with mean ± SEM. The sample size is shown in the bottom of each bar, and the solid five-pointed star indicates the calibrator used to normalize the gene expression. Data were analyzed by independent samples *t*-test. Significant differences between groups are indicated by asterisks (* $P< 0.05$, ** $P< 0.01$).

FIG 3 Knockdown of *nkd* gene in adult bees by dsRNA ingestion. All groups of adult bees were inoculated with *Nosema* spores first and then fed with sucrose solution

containing dsRNA for 15 days. The silencing effect was examined after 9 days (A) and 15 days (B) feeding of dsRNA, respectively. The control bees (GFP-dsRNA) were fed with the dsRNA derived from GFP sequence. For the treatment groups, three different concentration gradients of *nkd* dsRNA were examined. They were as follows, 40 μg/ml (nkd-dsRNA-40), 20 μg/ml (nkd-dsRNA-20), and 10 μg/ml (nkd-dsRNA-10). The relative gene expression values are shown as mean ± SEM. All groups are with the same sample size (n = 3). The calibrator used to normalize the gene expression is indicated with the solid five-pointed star inside the bar. One-way ANOVA was employed to analyze the differences of data, and post-hoc effects were identified by Tukey HSD tests. Significant differences between groups are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$).

FIG 4 Effect of *nkd* gene silencing on the immune gene expression in *Nosema* infected bees. The relative mRNA levels of immune genes (X axis) were compared between control bees fed with 20 μg/ml GFP dsRNA (GFP-dsRNA, n = 6) and the treatment bees fed with 20 μg/ml *nkd* dsRNA (nkd-dsRNA, n = 5). Both groups were inoculated with *Nosema* spores before dsRNA feeding. The expression values are expressed with mean ± SEM. Data were analyzed by independent samples *t*-test. Significant differences between groups are indicated by asterisks (* $P< 0.05$, ** $P< 0.01$).

FIG 5 Effect of *nkd* gene silencing on the *Nosema* infection levels in adult bees. The *Nosema* infection levels were determined by spore counting. Spore loads of the two groups of bees were compared, the control bees fed with 20 μg/ml GFP dsRNA (n = 11), and the treatment bees fed with 20 μg/ml *nkd* dsRNA (n = 8). The values of spore load are expressed with mean ± SEM. Significant differences of data were analyzed by independent samples *t*-test, and indicated with asterisks (* P< 0.05).

FIG 6 Effect of *nkd* gene silencing on the lifespan of honey bees infected by *N. ceranae*. Survival curves for bees inoculated with 100,000 *N. ceranae* spores at day 0 (i.e., within 24 h after adult emergence) and fed with 50% (m/V) sucrose solution containing 20 μg/ml *nkd* dsRNA (nkd-dsRNA, n = 60) or 20 μg/ml GFP dsRNA (GFP-dsRNA, n = 60) for 15 days, and for the bees without any treatment and only fed with 50% sucrose solution (No treatment, n = 60). Knockdown of *nkd* gene reduced the incidence of death (P = 0.041 by Wilcoxon test).